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Influence of L-Thyroxine Administration on Poor-platelet Plasma VEGF Concentrations in Patients with Induced Short-term Hypothyroidism, Monitored for Thyroid Carcinoma

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Abstract. Angiogenesis is a process of new blood vessel development from pre-existing vasculature. It is a crucial process in normal physiology, as well as in several pathological conditions. The vascular endothelial growth factor (VEGF) represents a family of specific endothelial cell mitogens, involved in normal angiogenesis and in tumour development. The aim of the present study was to estimate the influence of L-thyroxine (L-T_4) administration on poor-platelet plasma (P-PP) VEGF concentrations in patients with induced short-term hypothyroidism, monitored for differentiated thyroid carcinoma. In the present study, P-PP concentrations of VEGF, thyroglobulin, thyrotropin and free thyroid hormones were investigated in a population of 24 hypothyroid patients, who were withdrawn from L-T_4 treatment for 5 weeks and studied before and after 2 months of L-T_4 therapy. Only healthy female patients with no evidence of metastasis in whole body scintigraphy were included in the study. They were then compared with 20 healthy control subjects, matched for age, sex and body mass index (BMI). The patients had significantly lower plasma VEGF concentrations before treatment with L-T_4 than after administration of that hormone. There was no significant difference in plasma VEGF levels, either between the patients treated with L-T_4, and the controls, or between the patients untreated with L-T_4, and the controls. Even short-time changes in thyrometabolic profile exert an important influence on P-PP VEGF concentrations, even if there is no thyroid tissue.

Key words: VEGF, L-Thyroxine, Hypothyroidism

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ANGIOGENESIS is a proliferation of endothelial cells with their organisation into new blood vessels and/or a formation of new blood vessels from pre-existing microvasculature. It occurs in physiological and reparative conditions [1–4]. Angiogenesis is also implicated in several pathological conditions, such as inflammation and tumour growth [1–4]. Taking into consideration that angiogenesis is of central importance in tumour growth and progression, it has become a target in cancer therapy [5].

The vascular endothelial growth factor (VEGF) represents a family of specific endothelial cell mitogens — glycoproteins with potent angiogenic, mitogenic and vascular permeability-enhancing activities [1–4]. This glycoprotein has been implicated in tumour growth and has been proposed as a prognostic marker in several neoplasms [6–18]. Recently, VEGF expression has been observed in thyroid carcinoma, and VEGF production by neoplastically transformed thy-

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rocytes has been proven, both *in vitro* and *in vivo* [19–22]. VEGF expression is promoted by hypoxia, oestrogen, and nitric oxide [4]. On the other hand, its expression is suppressed by several factors, including retinoids, angiostatin, and corticosteroid [4]. Sato *et al.* reported that TSH and thyroid-stimulating antibodies increased the expression of VEGF mRNA *in vitro* in thyroid epithelial cells [26, 27]. Moreover, VEGF stimulated vascular endothelial cells in the thyroid, which resulted in a growing number of blood vessels and increasing thyroid volume. Conflicting results were obtained by Miyagi *et al.*, while investigating the influence of TSH on VEGF expression by FRTL-5 cells [28]. Furthermore, as demonstrated by Sorvillo *et al.* [29], a short-term administration of recombinant human TSH (rhTSH) in patients, monitored for differentiated thyroid carcinoma, induces a significant reduction in serum VEGF values, even in the absence of thyroid tissue. However, the results of the study [28] are discordant with those of Tuttle *et al.* [30]; thus, whether thyroid hormones and TSH stimulate VEGF production or not, remains still a controversial matter. In the present study, poor-platelet plasma (P-PP) VEGF, and serum thyroglobulin (Tg) and thyrometabolic statuses were investigated in a population of 24 hypothyroid patients, withdrawn from L-thyroxine (L-T_4) treatment for 5 weeks and (i) studied before and after 2 months of L-T_4 therapy and (ii) compared to 20 control subjects, matched for age, sex and body mass index (BMI).

Patients and Methods

Venous blood samples, collected from patients, thyroidectomized because of differentiated thyroid carcinoma, were submitted to the analysis. The patients were recruited from the Department of Endocrinology and Metabolic Diseases, Medical University of Lodz. All of them signed their informed consent, and the Ethics Committee of the Medical University of Lodz had approved the study protocol. Thyroidectomies — because of differentiated thyroid cancer — were performed 1–3 years before the study. Thyroidectomized patients were withdrawn from previous suppressive therapy with L-T_4 for, at least, five (5) weeks. Blood samples were collected in citrate tubes for plasma analysis. Venous blood was obtained by clean venipuncture, avoiding slow flowing draws and/or traumatic venipunctures. Needle gauge 19 was used. Blood samples were centrifuged at 180 RPM × g for 10 min. The centrifugation was performed 30 minutes after sample collection. After removing the supernatant, the samples were again centrifuged at 1500 RPM × g for 15 min to obtain platelet-poor plasma (P-PP). Plasma samples with signs of haemolysis were eliminated from further analysis. The samples were stored at –80°C until measurement of VEGF. The samples were collected at two time points: (i) at the time of the, so-called, stimulation with endogenous thyrotropin (TSH), *i.e.*, when the patients, remaining in the hypothyroid state ($n = 24$) were subjected to whole-body scintigraphy (WBS) and (ii) during 2 months of L-T_4 administration (3–4.5 μg/kg/day) in order to suppress TSH concentration. The patients with either signs of metastases on WBS or with immunological or metabolic disorders (*i.e.* diabetes mellitus) were excluded from the experimental protocol. The control subjects (free from metabolic disorders and age- sex- and BMI-matched with the patients) were recruited from among the University staff and their relatives. Those with thyroid disorders, increased goitre, increased Tg or increased antithyroid antibody levels were excluded.

Free triiodothyronine, FT_4, TSH and Tg concentrations were measured, using the immunoradiometric (IRMA) method with appropriate kits (BRAHMS, Berlin, Germany; normal values: TSH 0.3–4.0 mIU/l; FT_3, 2.2–5.0 pg/ml; FT_4, 10–25 pmol/l).

VEGF measurement was performed on duplicate aliquots of platelet-poor plasma, using a quantitative ELISA for human VEGF, according to the manufacturer's directions — R&D Systems, Minneapolis (sensitivity <5.0 pg/ml, intra-assay precision of 7.3% and inter assay precision of 5.4%). Sample readings were compared with positive controls (a standard curve, generated, using recombinant human VEGF) and negative controls (blank wells).

Statistical analysis

Student's *t* -test for paired samples was used to determine the significance of differences in all the measured parameters with normal distribution, observed between patients before and during L-T_4 therapy. Student's *t*-test for unpaired samples was used to determine the significance of the differences in all the measured parameters with normal distribution, be-

tween hypothyroid patients and control subjects, as well as between the L-T_4-treated patients and the controls. For TSH, FT_4 and FT_3, the data were not normally distributed and nonparametric Wilcoxon's rank test (for paired samples) and Mann-Whitney's test (for unpaired samples) were used to determine the statistical significance of differences.

Results

Laboratory data for the patients before and after treatment are shown in Table 1. The patients, selected for the study, before the treatment, presented initial serum TSH levels higher than the control subjects (51.25 ± 20.3 mIU/l *vs.* 1.12 ± 0.520.39 ± 0.53 mIU/l, respectively; $P<0.0001$) and were age-, sex- and BMI-matched with them. All the patients had evident thyroid hormone deficiency, with a few of them showing very high TSH levels. At the same time, serum FT_3 and FT_4 levels were significantly and markedly lower in hypothyroid patients than those in the control subjects (FT_3: 1.21 ± 0.48 pg/ml *vs.* 3.39 ± 0.75 pg/ml, $P<0.0001$; FT_4: 7.8 ± 2.18 pmol/l *vs.* 11.2 ± 3.6 pmol/l, $P<0.0001$). During 2 months of L-T_4 therapy, as expected, FT_3 and FT_4 concentrations were increased and TSH levels decreased (TSH: 0.39 ± 0.53 mIU/l; FT_3: 5.9 ± 1.66 pg/ml; FT_4: 27.98 ± 8.04 pmol/l; $P<0.0001$ *vs.* before L-T_4 therapy for all the parameters). Serum Tg concentration, although higher in the group before the therapy, did not differ significantly between the two groups (Table 1).

Table 1. Thyrometabolic state, serum Tg and P-PP VEGF concentrations in patients before and after L-T_4 treatment and in controls. Data are presented as means ± SD.

	Before L-T_4 therapy (n = 24)	After L-T_4 therapy (n = 24)	Controls (n = 20)
FT_4 (pmol/l)	7.8 ± 2.18	27.98 ± 8.04[ab]	11.2 ± 3.6
FT_3 (pg/ml)	1.21 ± 0.48	5.9 ± 1.66[ab]	3.39 ± 0.75
TSH (mIU/l)	51.25 ± 20.3	0.39 ± 0.53[ab]	1.12 ± 0.52
Tg (ng/ml)	1.67 ± 2.79	0.70 ± 0.70	2.0 ± 0.9
VEGF (pg/ml)	27.75 ± 4.32	31.93 ± 4.56[a]	32.5 ± 9.65

[a] p>0.005 *vs.* before L-T_4 treatment
[b] p>0.005 *vs.* controls

P-PP VEGF concentrations

The patients, untreated with L-T_4, had significantly lower P-PP VEGF concentrations than after treatment, as shown in Fig. 1 and Table 1. There was no significant difference in PPP VEGF levels between the patients after treatment with L-T_4 and the controls. Plasma VEGF levels in those patients increased significantly after treatment (Fig. 2). Weak — but still significant — correlations between plasma VEGF and F-T_3 levels ($r = 0.465$; $p<0.005$) and between VEGF and FT_4 concentrations ($r = 0.444$; $P<0.01$) were observed. Neither TSH nor Tg levels correlated with VEGF P-PP concentrations.

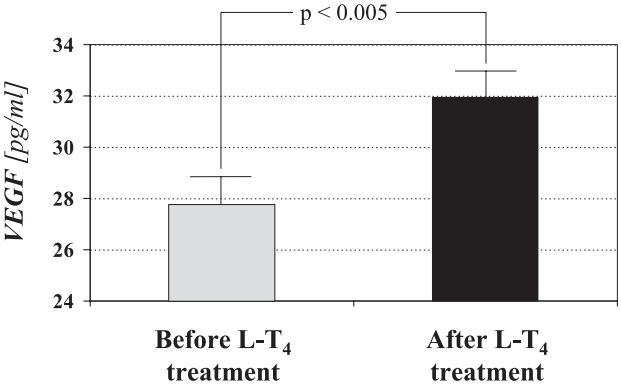

Fig. 1. Concentration of P-PP VEGF before and after L-T_4 treatment. Data are presented as means ± SEM.

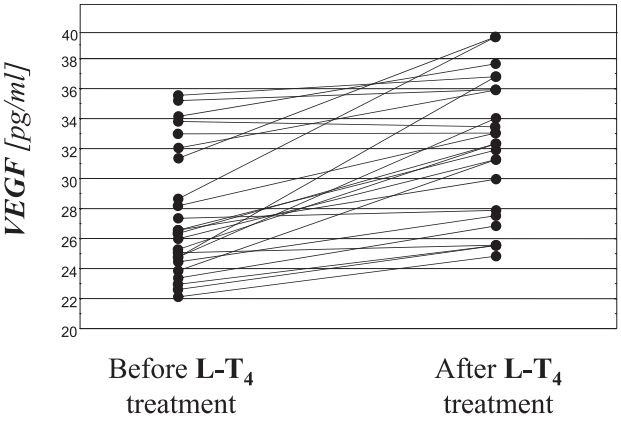

Fig. 2. Changes of P-PP VEGF concentrations during the L-T_4 treatment in particular patients — before and after L-T_4 treatment.

Discussion

The type of sample, which should be used in VEGF measurements, is still a matter of debate.

Most of the recent studies on the influence of TSH and thyroid hormones on VEGF concentrations are performed on serum samples [25, 29, 30]. The results of some reports on circulating VEGF, performed with the use of serum samples, suggest that serum could be unsuitable for sampling VEGF [31–34]. It has been demonstrated that VEGF is stored in granules and released on platelet activation during clotting [36, 41]. Platelets represent the main reservoir of circulating blood VEGF and it has been demonstrated that platelets can endocytose and concentrate circulating plasma VEGF [37, 38]. Although various blood cells, such as granulocytes, monocytes, mast cells and lymphocytes, have been shown to be capable of producing VEGF, these cells are of little importance for the release of VEGF into circulation [39–41]. Therefore, serum VEGF may be an inaccurate indicator of circulating VEGF and thus, P-PP plasma is recommended for the measurement of circulating extracellular VEGF [37, 38]. In turn, other authors suggest that platelet-derived VEGF also reflects the biology of cancer cells — the platelets may scavenge tumour-cell-released angiogenic stimulators and inhibitors from tumour vasculature — and serum should be used for the measurement of VEGF levels in cancer patients [42]. Since, in the present study, only those patients were considered who had been free from cancer diseases, we decided to analyse P-PP samples. This is, to our knowledge, the first study, analysing changes in VEGF P-PP concentration caused by L-T_4 administration.

Our study has demonstrated that short-term hypothyroidism in patients, monitored for thyroid carcinoma, induces a significant reduction in plasma VEGF levels. This observation is not concordant with some recent results [43, 44]. However, it is noteworthy that in our experiment VEGF reduction occurred in those patients who did not show either any biochemical or morphological presence of thyroid tissue. In several current studies, the authors have analysed changes of VEGF expression or serum concentration in different pathologies of the thyroid gland, including autoimmune thyroid diseases and cancers [19–30]. VEGF expression appears to be related with tumour behaviour. Higher VEGF expressions are present in metastatic thyroid cancer, when compared with nonmetastatic disease [30]. Moreover, higher VEGF expression correlates with tumour size in adults and children [45].

Klein *et al.* have shown that VEGF is weakly expressed in normal thyroid tissue, while revealing strong expression in thyroid carcinoma, as well as in thyrocytes from patients with chronic lymphocyte thyroiditis [19], and that increased expression of VEGF is a preoperative marker in papillary thyroid carcinoma [46]. Also serum VEGF is elevated in patients with untreated Graves' disease and Hashimoto's thyroiditis [47]. Despite the fact that the patients included in the above cited study were either hypo- or hyperthyroid, we cannot directly refer them to our results, considering the autoimunological character of both diseases. Recently, Hoffmann *et al.* have demonstrated that TSH increases the expression of VEGF mRNA in thyroid cancer cell lines [48]. In an earlier experiment, TSH and Graves' IgG increased VEGF levels in human thyroid follicles *in vitro* and increased constitutive VEGF secretion by thyroid cells in culture [26]. Opposite results were obtained by Miyagi *et al.*, using FRTL-5 [28]. However, VEGF expression was determined in different experimental conditions, which may, at least in part, explain the difference in question. Studies on thiouracil-induced hypothyroidism have demonstrated that elevated TSH leads to increased VEGF concentrations, which subsequently resulted in VEGFR expression [26]. However, Tuttle *et al.* [30] did not observe any difference in VEGF serum concentrations in patients before and after stimulation with recombinant human TSH.

On the other hand, Suzuki *et al.* have reported that thyroglobulin regulates thyroid specific gene expression, including VEGF. Moreover, they have reported that the effect of Tg is much stronger than that effect of TSH [49].

In our study, the changes of VEGF concentration resulted exclusively from L-T_4 administration and direct changes of hormone profile, induced by that treatment. We have used a generally known clinical model, in which VEGF concentrations can be investigated in the same patient in two different thyrometabolic states — short-term hypothyroidism and short-term subclinical thyrotoxicosis. The patients included in the experimental protocol, were free from any metabolic or immunological disease. Thus, they presented an almost perfect model to analyse the influence of L-T_4 administration on the profile of selected molecules. The effect of Tg in our experimental conditions was also limited.

Although we observed a difference in Tg levels between both groups, it did not reach the border of significance; neither any correlation was observed between Tg and VEGF concentrations. The temporal relation between L-T_4 administration and VEGF increase is, in those cases suggestive of a specific effect. However, L-T_4-treatment withdrawal and readministration caused direct and indirect changes in the thyrometabolic hormone profile. Therefore, the changes in VEGF P-PP concentrations most probably resulted from the simultaneous action of TSH, Tg and thyroid hormones. Similar results were obtained by Schmid *et al.* who investigated the influence of L-T_4 replacement therapy on serum VEGF concentrations in primary hypothyroid patients [48]. They observed increase of VEGF serum concentration during the replacement therapy. However, since they analysed VEGF concentrations in serum, they could not exclude that the observed changes are the effect of increased VEGF release from platelets [51, 52]. In conclusion, our results suggest that, even in thyroidectomized patients, the thyrometabolic profile affects P-PP VEGF concentration in an important way. The present study did not give us any opportunity to demonstrate the mechanism responsible for VEGF decrease after L-T_4 treatment withdrawal, which probably will be the subject of our further studies. However, in our opinion, while VEGF concentration is investigated, the thyrometabolic state of the patient should be considered. Moreover, the increase of P-PP VEGF concentration in response to L-T_4 administration may, at least in part, improve endothelial function and renal blood flow, thereby contributing to the decrease of serum creatinine concentration, observed in hypothyroid patients treated with L-T_4 [50].

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